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*Michael M. Walsh*      *9/25/97*  
PI - Signature      Date

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## Introduction

Ataxia-telangiectasia (AT) is a heritable syndrome in which carriers appear clinically normal but may have an elevated risk of developing breast cancer. The work originally proposed in this application was designed to determine if ATM, the gene mutated in AT, acts as a breast cancer tumor suppressor gene. The approach was to survey breast cancers for tumor loss of constitutional heterozygosity for DNA markers in 11q22-q23, the chromosomal region where ATM maps.

During the first year of the project, 37 sets of tumor and normal tissue in pathology storage as paraffin embedded samples were screened for 14 polymorphic loci based on simple sequence repeats (SSRs) using the polymerase chain reaction (PCR). Seventeen of these tumors were found to have lost heterozygosity either in the region of ATM or an adjacent region that might overlap. All but one of these deletions appear to be interstitial.

In mid-1995 a partial cDNA derived from the ATM gene was cloned by Shiloh and his colleagues (Savitsky et al. 1995). The cloning of ATM presented an opportunity to directly test breast cancer patients for mutations in this gene. During the August 1995 to August 1996 funding period we began the development of assays to detect ATM mutations. Since the entire gene was not yet cloned we started by generating antibodies to ATM protein. Rabbit polyclonal antiserum was generated against a recombinant ATM protein produced in a bacterial expression system. This antiserum was tested for reactivity with ATM protein by Western blotting, immunohistology and immunoprecipitation and found to be useful only for immunoblotting. In view of this limitation the antiserum was not suitable for accomplishing the goals of this project.

As soon as the complete coding sequence of the gene was available we began development of an assay for the detection of AT carriers using RNA isolated from peripheral blood lymphocytes. The basis of this assay was the restriction endonuclease fingerprinting (REF) procedure (Liu and Sommer 1995). REF is a variation of SSCP (single strand conformational polymorphism) analysis modified for long stretches of sequence and, like SSCP, it is capable of detecting single base changes that do not result in a truncated protein product. Our adaptation of REF to ATM was validated by testing lymphoblastoid cell lines derived from individuals who are normal, obligate AT heterozygotes and AT homozygotes. The results provided preliminary evidence that REF can identify sequence variations in the ATM transcript as defined by SSCP.

The granting period for this project was extended for one year (August 1996 to August 1997), without the allocation of additional funds, to confirm the REF results by DNA sequencing and to initiate an epidemiological study on the effects of ATM mutations and polymorphisms on breast cancer risk and treatment. Both of these goals have been accomplished in the final funding period and are described below.

## Body

### Assay Development

#### *Overview*

The detection of mutations and polymorphisms in the ATM gene presents a considerable technical challenge for a number of reasons. One is the large number of exons comprising the gene and the sheer size of the ATM transcript. The ATM gene consists of at least 65 exons and is transcribed and processed into a 12 kb mRNA of which just over 9 kb encodes amino acid sequence. Naturally occurring variations that might result from differential splicing or a highly variable 5' UTR are not yet well characterized. In addition, the mutations described so far tend to be single base pair changes and small insertions or deletions that are scattered through the coding region. Over 80% of the known ATM mutations result in protein truncations and this has led to suggestions that the in vitro protein truncation test (PTT) could be used as a primary screen for ATM mutations (Telatar et al, 1996). However, almost all of the known mutations were characterized from individuals with AT or their relatives and it is by no means clear that the mutations that cause clinical AT are the only ones that could lead to cancer susceptibility or radiosensitivity. As broader populations are screened more subtle mutations will emerge including mutations that result in single amino acid changes which are not detected by PTT. In some cases these may be more accurately described as polymorphisms than mutations. Consequently, we have decided not to use PTT in these studies despite its relative simplicity when compared to other assays.

The most rigorous approaches for ATM mutation detection reported to date are PCR-SSCP analysis of genomic DNA (Vorechovsky et al. 1996) and REF analysis of the message (Savitsky et al. 1995). Application of the PCR-SSCP assay to large numbers of samples of a 65 exon gene is a massive and expensive undertaking, although multiplexing and eliminating some exons as regions that could harbor consequential mutations may one day make it the method of choice. REF analysis of a 9 kb mRNA is by no means trivial, but REF analysis is slightly less cumbersome than PCR-SSCP for long stretches of sequence and has the advantage of a higher detection rate. A disadvantage is the use of RNA rather than DNA as the starting material.

In the previous funding period we tested REF as a method to detect ATM mutations and polymorphisms. Using RNA from cell lines derived from AT patients and their parents we identified several potential mutations. We proposed to confirm these by sequence analysis.

#### *RNA Preparation and RT-PCR*

Lymphocytes were isolated from peripheral blood by centrifugation on ficoll hypaque. Total lymphocyte RNA was prepared by the method of Chomczynski (Chomczynski and Sacchi 1987) using commercially available reagents (RNAzol B, Tel-Test, Friendswood, TX). Total RNA was reversed transcribed with random primers and the ATM cDNA subsequently amplified

by PCR. The PCR primer sets were designed to amplify ATM cDNA as 8 overlapping products ranging in size from 1200 to 1600 basepairs.

### *cDNA Sequencing*

Agarose gel purified RT-PCR products were sequenced using commercially available cycle sequencing methodology (Amersham). The PCR primers double as sequencing primers. Elimination of background bands is crucial to the detection of a hemizygous base change. We have tested both terminal deoxynucleotidyltransferase extension of the sequencing reaction products and the use of <sup>32</sup>P-labeled chain terminators to reduce background. In our hands, labeled chain terminators gave better results and this procedure is now standard in the assay.

A Genomyx-LX sequencing apparatus is used for electrophoretic resolution of the sequencing products. Each reaction is assayed on two gels, the first designed to resolve from the primer to 350 bases and the second designed to resolve out to 800 bases. In actual practice we can resolve more than 800 bases from a single sequencing reaction. The RT-PCR products are 1600 basepairs or less so, with this system, each product can be completely sequenced using two sequencing reactions (one from either end) and two gel runs. Following electrophoresis, the dried sequencing gels are exposed to x-ray film for detection. For our purposes AT heterozygotes include individuals carrying any ATM sequence other than the GenBank reference sequence, HSU33841.

### *ATM Sequencing Results*

Previously we used REF analysis to search for alterations in ATM mRNA from 12 lymphoblastoid cell lines established from individuals with AT or obligate heterozygotes. Anomalous REF banding patterns were detected in 10 of these lines. These results suggest REF analysis may miss some ATM mutations. We used the sequencing assay to determine the mutations in three of the anomalous REF fragments (Table 1). In doing so, we found that direct sequencing was less time consuming than REF. Consequently, direct sequencing of all eight PCR products has replaced REF for ATM mutation/polymorphism detection in this laboratory.

We next confirmed that the direct sequencing assay would detect ATM mutations in peripheral blood samples from patients. Blood samples were provided by two obligate ATM heterozygotes for analysis. In both cases cDNA sequencing identified changes in the ATM mRNA of these individuals (Table 1 and Figure 1). In one case the cDNA alteration resulted in the formation of a stop codon that eliminates the PI-3 kinase domain of the ATM protein, and in the other the resulting amino acid change occurs in the region of the PI-3 kinase motif (Morgan et al., 1997). This alteration, resulted in the substitution of a cysteine residue for an arginine residue.

The direct sequencing assay is now being used to detect ATM mutations and polymorphisms in patients recruited for Protocol R0 97-034 (discussed below). Currently, 50 samples have been submitted for analysis and 6 of these have been sequenced in their entirety.

Three missense mutations or polymorphisms have been detected (Table 1 and Figure 1) in these 6 completed samples.

### Establishment of a Collaborative Project

#### *Overview*

The assay described above can detect ATM mutations and polymorphisms and that allows the design of epidemiological studies to resolve the clinically important questions about ATM carrier status. Some of these questions are:

- Do ATM mutations/polymorphisms increase susceptibility to cancer, particularly breast cancer?
- Do ATM mutations/polymorphisms increase the risk of complications from radiotherapy?
- Are tumors arising in AT heterozygotes more radiosensitive than comparable tumors in other individuals?

To actually carry out these studies requires the cooperation of individuals representing several disciplines. We have established an ATM Working Group that encompasses the necessary expertise. This group has secured approval for a clinical protocol from the M.D. Anderson Surveillance Committee and has initiated a pilot study of ATM mutation incidence in cancer patients.

#### *ATM Working Group*

The members of the ATM Working Group are listed in Table 2.

#### *Clinical Protocol*

Protocol RO 97-34 was activated in April 1997. The study chairman is Eric A Strom, M.D. of the Department of Radiation Oncology. Under this protocol, study participants provide a blood sample for ATM testing. Patients with invasive cancer and individuals who have an exaggerated response to previously delivered irradiation (RTOG grade III and above) are eligible for the study.

#### *Pilot Study*

Protocol RO 97-34 allows us to screen 250 patients for ATM status. The goal is to determine if the incidence of ATM mutations and polymorphisms in this population is high enough to warrant a large scale epidemiological study. We have already accrued about 50 individuals, most of them breast cancer patients. Analysis of their ATM status is on-going.



## **Conclusions**

1. A direct sequencing assay that allows the detection of mutations and polymorphisms over the entire coding sequence of ATM mRNA has been developed and implemented. The assay can be run on peripheral blood samples.
2. A pilot study to determine the incidence of ATM mutations and polymorphisms in 250 cancer patients has begun. If this pilot study is successful it will be expanded to a full scale epidemiological study of the role of ATM mutations and polymorphisms in breast cancer susceptibility and normal tissue damage from radiotherapy.

## **Personnel Receiving Pay From This Effort**

Michael M. Weil, Ph.D.	Principal Investigator
Yue Lin, M.S.	Research Assistant II

## **Meeting Abstracts**

Chromosome 11q Loss of Heterozygosity in Breast Cancer  
DOD Breast Cancer Research Program: An Era of Hope  
10/31/97 - 11/4/97  
Washington D.C.

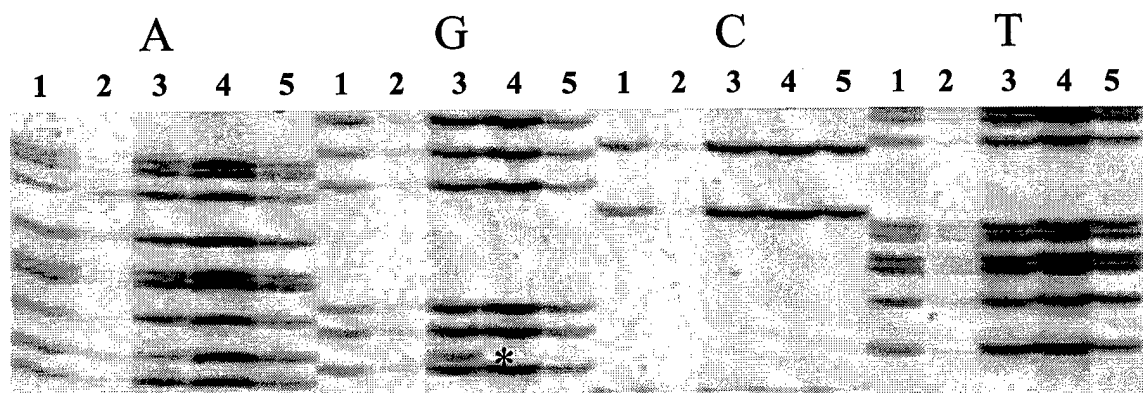
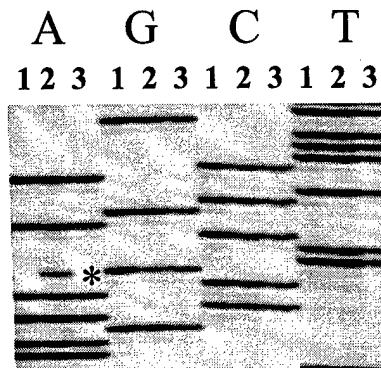
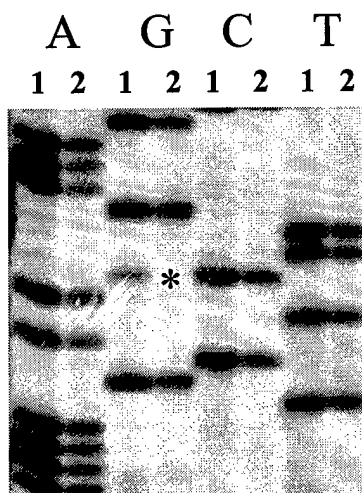
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## Appendix

**TABLE 1**

<b><u>Source</u></b>	<b><u>ATM status</u></b>	<b><u>Alterations</u></b>
Cell line (GM 334A)	heterozygote	Gly 514 > Asp Ala 554 > Thr
Cell line (GM 11261)	homozygote	Arg 2443 > Ter Arg 2486 > Gln
Blood	obligate heterozygote	Tyr1124 > Ter Asp1853 > Asn
Blood	obligate heterozygote	Arg 3008 > Cys
Blood	study participant	Pro 1054 > Arg
Blood	study participant	Ser 707 > Pro
Blood	study participant	Asp1853 > Asn



**Detection of mutations in ATM in samples from two obligate AT heterozygotes and from a breast cancer protocol participant.** **Upper left panel:** region of sequencing gel identifying an alteration in cDNA coding sequence in one allele. The left lane of the paired samples is from the obligate carrier, the right lane is from a normal control. In this case the alteration has resulted in the substitution of a termination signal for tyrosine 1124. **Upper right panel:** region of sequence identifying a missense mutation that substitutes cysteine for arginine 3008. This mutation would not have been detected by a protein truncation assay. The middle lane is that of the obligate carrier. The adjacent lanes are from normal controls. **Lower panel:** region of ATM sequence from a sample provided by a participant in the breast cancer protocol. The sample in lane 3 has a single base change in one allele that substitutes a codon for proline in place of that for serine 707.

## **TABLE 2**

### **MDACC ATM Working Group**

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